ABSTRACT

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), the superoxide radical (O_2^-), and the hydroxyl radical (OH^-) are produced in plant cells during physiological metabolic processes such as photosynthesis and respiration. ROS production is also induced in plants under both biotic and abiotic stress, including high salinity conditions, drought, intense light, high concentration of heavy metals in the soil and microbial attack. ROS are cytotoxic and, due to their increased activity, interact with macromolecules such as proteins, lipids and nucleic acids, causing irreversible damages which can lead to cell death. In order to cope with ROS accumulation, plants have evolved defense mechanisms of enzymatic or non-enzymatic nature to scavenge or block the production of these hazardous molecules. In addition to the hazardous effect, ROS also play signalling functions within cells and participate in several developmental and functional processes in plants. Thus, besides from scavenging ROS, antioxidative enzymes are responsible for maintaining redox balance in the cell.

Phospholipid hydroperoxide glutathione peroxidases (PHGPXs) are members of the antioxidant mechanism, metabolizing organic hydroproxides or H_2O_2 to water with the use of an electron provided by either glutathione or thioredoxin. PHGPXs are highly conserved and exhibit certain characteristics such as their subcellular localization, which in plants is usually both cytosolic and mitochondrial and depends on an N'-terminal leading peptide. They do not form homopolymers, and their genomic sequences are comprised by six, seven or eight exons. Finally, PHGPXs would also act as redox state sensors and signal transducers, other than ROS scavenging enzymes.

Nitric oxide (NO), as well as H_2O_2, are positive regulators of abiotic- and biotic-induced stress defense response in plants. NO is a hydrophobic gaseous molecule that has the ability to easily and rapidly diffuse between and within cells. It interacts with metal complexes, proteins and nucleic acids and is implicated in several physiological procedures like lateral root elongation, stomatal closure, apoptosis and response to biotic and abiotic stresses. It can also be coupled to a reactive cysteine thiol, forming *S*-nitrosothiol (SNO), a post-translational modification

that modifies the enzymatic activity of target enzymes. NO can interact with ROS to form reactive nitrogen species (RNS) such as peroxinitrite, a molecule which causes the post-translational modification of tyrosine nitration.

The aim of this thesis was to analyze the existence of putative PHGPX homologues in *M. truncatula*, characterize them and study their response to NO on a transcriptional and protein level. To this end, two PHGPX isoforms (*MtPHGPXα* and *MtPHGPXβ*) were isolated and compared to other known PHGPX from other species. They were found to be containing all conserved regions and two alternatively spliced leading peptides. In order to investigate whether these peptides are responsible for the subcellular localization of the isoforms, the full length gene and the two coding regions of *MtPHGPXα* and *β* were fused to GFP protein tags and overexpressed in tobacco leafs. Both isoforms were found to be located in the cytosol whereas the full length gene construct also gave a protein which was located in the cytosol as well. The effect of NO and H_2O_2 on the representation of the two transcripts was also studied in roots and leaves and different patterns were observed. To elucidate the mechanism by which NO could control MtPHGPX levels an mRNA stabilization assay was performed, which showed that NO could extend *MtPHGPX* transcripts half-life.

Another objective was to specify the substrate and the electron donor used by MtPHGPX. The protein was overexpressed in bacteria and purified. It was found that the substrate and electron donor with the greatest affinity was H_2O_2 and thioredoxin, although cumene hydroperoxide showed similar affinity. NO was not found to influence MtPHGPX activity but peroxynitrite showed a strong inhibition effect. MtPHGPX cystein residues were shown to be *S*-nitrosylated by NO donor DEANONOate and tyrosine residues were found to be nitrated by peroxynitrite. Finally, a protein-protein interaction assay showed that MtPHGPX could possibly interact with an unknown protein of the plant, an interaction which is probably inhibited by H_2O_2.

This work gave insight into two MtPHGPX isoforms and their responses to oxidative stress simulation conditions mimicked by NO and H_2O_2. It was found that these isoforms are products of alternative splicing and their representation is controlled by NO and H_2O_2 in roots and leaves. The isoforms are closely related to

other legume species and contain all consereved regions except for two leading peptides alternatively present. Interestingly both isoforms were found to be cytosolic, an observation which seems to be common for legume plants.

The activity of the enzyme was not found to be affected by NO, but it was strongly inhibited by peroxynitrite. This could mean that NO is not bound to a cystein that is crucial for the formation and the function of the active site of the enzyme, whereas a tyrosine residue must be very important. This could also point towards an NO binding role served by MtPHGPX and when combined with the fact that both isoforms are cytosolic a functional role could arise. MtPHGPX might be able to protect nitrogenase in nodules from inhibitory excess NO levels, in parallel with ROS scavenging, which has the same effects.

As future perspectives, these results could be very useful for further investigation in line with the broad research field on the antioxidant mechanism and dealing with oxidative stress. The information on MtPHGPX could be proven helpful to develop new approaches towards a more efficient yield policy. Nevertheless, the specification of the exact mechanism by which MtPHGPX transcripts are accumulated in the cell and the identity of the protein interacting with MtPHGPX are very interesting issues to be studied. The effect of NO on the stability of the protein and the functional role of the enzyme are also considered to be of high importance.